CLAIMS

1. A method for in *vitro* immuno-diagnosis of antigen-specific T lymphocytes based on the preparation of compositions, also called stimuli, able to stimulate the T lymphocytes; such compositions comprising at least one among the antigens in different forms selected in the group of: (a) raw protein extract, (b) purified or recombinant proteins, (c) synthetic peptides and combinations of (a), (b) and (c); such stimuli being identified as pathogen-specific when based on antigens originating from pathogens and vaccine-specific when based on antigens originating from strains used for making vaccines they; said method invention comprising the following steps:

i) isolation of peripheral blood mononuclear cells (PBMC) from a sample of human or animal venous blood;

ii) preparation of at least one stimulus selected between pathogen-specific and vaccine-specific stimuli,

iii) preparation of a negative control comprising cells cultivated in vitro in complete medium without stimuli and a positive control comprising cells cultivated in vitro in complete medium with an aspecific stimulus;

iv) stimulation of said T-lymphocytes with the vaccine-specific or the pathogen-specific stimulus in the presence of a costimulus;

v) incubation;

vi) selective staining by immunofluorescence;

vii) flow-cytometry acquisition and analysis;

viii) measurement and characterization of the immune response.

2. A method according to claim 1 where data evaluation and response are given by identifying a cut-off value for the specific response, set by common statistical methods as the average plus two times the standard deviation of the T cell response frequency obtained from a sample of healthy persons.

3. A method according to claim 1 where the aspecific stimulus is selected between phorbol myristic acetate and ionomycin.

4. A method according to claim 1 where PBMC are isolated from a sample of venous blood by centifugation on a density gradient.

5. A method according to claim 1 where the incubation in step v) is performed for one hour at 37° C in a humidified CO_2 incubator, followed by an incubation of at least 3 hours in the presence of an inhibitor of the cellular secretion.

6. A method according to claim 1 wherein said selective staining of antigen-specific (Ag-Sp) T lymphocytes in step (vi) is performed by:

   A) a monoclonal antibody against at least one T lymphocyte membrane antigens or subpopulation thereof;

   B) a monoclonal antibody against a cytokine

   C) a mixture of A) and B).

7. A method according to claim 6 wherein in item A) said T lymphocyte membrane antigens are chosen among: CD3, CD45, anti-CD4, CD8, CD25, CD27, CD38, CD45-RA, CD45-RO, CD69, CCR5, or CCR7.

8. A method according to claim 7 wherein said T lymphocyte membrane antigens are CD3 and CD45.

9. A method according to claim 6 wherein in item B) cytokines are selected from the group consisting of: interferon gamma, IL-2, IL-4, IL-10,TNF-α, MIP-1α, MIP-1β, RANTES, and corresponding mixtures.

10. A method according to claim 9 wherein said cytokine is interferon gamma.

11. A method according to claim 5 wherein said secretion inhibitor is selected between brefeldin-A and monensin.

12. A method according to claim 11 wherein said secretion inhibitor is brefeldin-A.

13. A method according to claim 1 wherein in step (iii) the co-stimulus is obtained by incubating the T-lymphocytes in the presence of an anti-CD28 and/or an anti-CD49d monoclonal antibody.

14. A method according to claims 1-13 to detect T- lymphocyte specific for infectious agents, tumor antigens, autoimmune antigens and allergenic agents.

15. A method according to claim 14 for *in vitro* diagnosis of infectious, autoimmune, allergic and neoplastic diseases.

16. A method according to claim 14 for detecting a resolution or a relapse of a pathology or for detecting the effectiveness of a chemotherapy or of a vaccination protocol.

17. Method according to claims 1-16 that is computer-made.

18. Sofware comprising the software paths that carry out the steps of the method claimed according to claims 1-16.

19. A method to design the peptides as in point (c) according to claim 1, said method comprising the following steps:

1) selection of a specific protein of a pathogen;

2) optionally definition of a "consensus sequence", accounting for any possible strain or clade or subtype pathogen heterogeneity;

3) definition of the HLA Class I-binding peptides by SYFPEITHY (http://syfpeithi.bmi-heidelberg.com/) or BIMAS (http://bimas.dcrt.nih.gov/molbio/hla_bind/) ;

4) selection of the peptides, with binding scores;

5) identification of immunodominant regions and of peptides which bind to at least two different HLA loci (HLA-A and -B, or HLA-A and -C, or HLA-B and -C), or preferably to all three loci (HLA-A and -B and -C);

6) identification of peptides of at least 9 aminoacid in length overlapping the immunodominant region;

7) selection of antigen-specific peptides by protein-protein BLAST (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi);

8) design of a peptide mixture or composition.

20. A method according to claim 19 wherein pathogens are selected among: Variola (Ortho-Poxviruses), Anthrax (*B.anthracis*), Plague (*Yersinia pestis*), Tularemia (*Francisella tularensis*) and SARS (Coronavirus).

21. A method according to claim 19 wherein for variola and coronaviruses, proteins are selected from the core, from the surface/envelope and from regulatory proteins

22. A method according to claim 19 wherein for bacteria, proteins are selected among toxins associated to pathogenicity.

23. Method according to claims 19-22 that is computer-made.

24. Sofware comprising the software paths that carry out the steps of the method claimed according to claims 19-22.

25. Composition of peptides comprising at least one of following groups of peptides:

Ortho-Poxvirus peptides from sequence ID84 to 85, from sequence ID86 to 87, peptides from sequence ID88 to 90, peptides from sequences ID91 to 92, peptides sequence ID93, peptides from sequence ID94 to 95, peptides from sequence ID96 to 97, peptides from sequence ID98 to 99, peptides from sequence ID100 to 101, peptides from sequence ID102 to 103;

Anthrax (*B.anthracis*) peptides from sequence ID74 to 83;

SARS coronavirus: peptides from sequence ID44 to 59, peptides from sequence ID45 to 46 and from ID60 to 61, peptides from sequence ID47 to 48 and from ID62 to 63, peptides from sequence ID49 to 58 and from ID64 to 73, peptides from sequence ID45 to 46;

Human non-SARS Coronavirus peptides from sequence ID173 to 177, peptides from sequence ID178 to 182.

26. A composition for detecting specific T-lymphocyte activation comprising at least three peptides selected from the group consisting of peptides comprising at least 9 consecutive aminoacids comprised within anyone of the peptides from SEQ ID NO 1 to SEQ ID NO 182.

27. The composition according to claim 26 for immunodiagnosis of HIV infection comprising at least three HIV gag peptides selected from the group consisting of peptides comprising at least 9 consecutive aminoacids comprised within anyone of the following peptides: SEQ ID NO 1 to SEQ ID NO 20.

28. The composition according to claim 27 comprising at least three HIV gag peptides selected in the group consisting of: SEQ ID NO 1 to SEQ ID NO 20.

29. The composition according to claim 26 for immunodiagnosis of CMV infection comprising at least three peptides selected from the group consisting of peptides comprising at least 9 consecutive aminoacids comprised within anyone of the following peptides: SEQ ID NO 21 to SEQ ID NO 43.

30. The composition according to claim 29 comprising at least three peptides selected in the group consisting of SEQ ID NO 21 to SEQ ID NO 43.

31. The composition according to claim 26 for immunodiagnosis of SARS coronavirus infection comprising at least three peptides selected from the group consisting of peptides comprising at least 9 consecutive aminoacids

comprised within anyone of the following peptides: SEQ ID NO 44 to SEQ ID NO 73.

32. The composition according to claim 31 comprising at least one peptide selected in the group consisting of SEQ ID NO 44 to SEQ ID NO 73.

33. The composition according to claim 32 comprising at least one of the SARS coronavirus E-protein derived peptide corresponding to SEQ ID NO 44 or to SEQ ID NO 59.

34. The composition according to claim 32 comprising at least three of the SARS coronavirus M-protein derived peptides corresponding to: SEQ ID NO 45, SEQ ID NO 46, SEQ ID NO ID60, SEQ ID NO 61.

35. The composition according to claim 32 comprising at least three of the SARS coronavirus N-protein derived peptides corresponding to: SEQ ID NO 47, SEQ ID NO 48, SEQ ID NO 62, SEQ ID NO 63.

36. The composition according to claim 32 comprising at least three peptides from each set of SARS coronavirus S-protein derived peptides wherein set 1) consists of SEQ ID NO 49 to SEQ ID NO 58 and set 2) consists of SEQ ID NO 64 to SEQ ID NO 73.

37. The composition according to claim 32 comprising at least one of the SARS coronavirus M-protein derived peptides corresponding to sequences: SEQ ID NO 45 and SEQ ID NO 46.

38. The composition according to claim 32 for immunodiagnosis of SARS infection specific for the Asian population (A-SARS) comprising at least three peptides from SEQ ID NO 44 to SEQ ID NO 58.

39. The composition according to claim 32 for immunodiagnosis of SARS infection specific for the Caucasian population (B-SARS) comprising at least three peptides selected from the group of : SEQ ID NO 59 to SEQ ID NO 73.

40. The composition according to claim 26 for immunodiagnosis of infectious diseases comprising at least two peptides selected from the group of peptides consisting of peptides comprising at least 9 consecutive aminoacids comprised within anyone of the following set of peptides: set 1) consisting of SEQ ID NO 1 to 20, set 2) consisting of: SEQ ID NO 21 to 43, set 3) consisting of: SEQ ID NO 44 to 73.

41. The composition according to claim 40 wherein said infectious diseases are AIDS, CMV and coronavirus (SARS) infections.

42. The composition according to claim 26 for immunodiagnosis of *B.anthracis* infection comprising at least three peptides selected from the group consisting of peptides comprising at least 9 consecutive aminoacids comprised within anyone of the following peptides: SEQ ID NO 74 to SEQ ID NO 83.

43. The composition according to claim 40 comprising at least three peptides selected from the group of: SEQ ID NO 74 to SEQ ID NO 83.

44. The composition according to claim 26 for immunodiagnosis of *orthopoxviridae* infection or vaccination comprising at least three peptides selected from the group consisting of peptides comprising at least 9 consecutive aminoacids comprised within anyone of the following peptides: SEQ ID NO 84 to SEQ ID NO 103.

45. The composition of claim 44 comprising at least three peptides selected from the group of: SEQ ID NO 84 to SEQ ID NO 103.

46. The composition according to claim 26 for immunodiagnosis of threat disease infections comprising at least two peptides selected from the group consisting of peptides comprising at least 9 consecutive aminoacids comprised within anyone of the following set of peptides: set 1 consisting of: SEQ ID NO 74 to 83, set 2 consisting of: SEQ ID NO 84 to 103.

47. A composition for immunodiagnosis of enteric infections comprising as immunostimulants comprising the following antigens: *Shigella* groups A, A1, B, C, C1, C2 antigens, *Salmonella* groups A, 0 antigens, Enterovirus 70 antigen lysate, HAV antigen lysate, HEV Hepatitis E Virus ORF2 antigen, *Helicobacter pylori* HPSa antigen, *Clostridium difficile* Toxin A antigen.

48. The composition according to claim 26 for detecting alpha-fetoprotein specific T-lymphocytes comprising at least three peptides selected from the group consisting of peptides comprising at least 9 consecutive aminoacids comprised within anyone of the following peptides: SEQ ID NO 104 to SEQ ID NO 122.

49. The composition according to claim 48 comprising at least three peptides selected in the group consisting of: SEQ ID NO 104 to SEQ ID NO 122.

50. The composition according to claim 26 for detecting of PSA specific T-lymphocytes comprising at least three peptides selected from the group consisting of peptides comprising at least 9 consecutive aminoacids comprised within anyone of the following peptides: SEQ ID NO 123 to SEQ ID NO 142.

51. The composition according to claim 50 comprising at least three peptides selected from the group of: SEQ ID NO 123 to SEQ ID NO 142.

52. The composition according to claim 26 for detecting MAGE-3 specific T-lymphocytes comprising comprising at least three peptides selected from the group consisting of peptides comprising at least 9 consecutive aminoacids comprised within anyone of the following peptides: SEQ ID NO 143 to SEQ ID NO 157.

53. The composition according to claim 52 comprising at least three peptides selected from the group of: SEQ ID NO 143 to SEQ ID NO 157.

54. The composition according to claim 26 for detecting NY-ESO-1 antigen specific T-lymphocytes comprising at least three peptides peptides selected from the group consisting of peptides comprising at least 9 consecutive aminoacids comprised within anyone of the following peptides: SEQ ID NO 158 to SEQ ID NO 172.

55. The composition according to claim 54 comprising at least three peptides selected from the group of : SEQ ID NO 158 to SEQ ID NO 172.

56. The composition according to claim 26 for immunodiagnosis of tumors comprising at least two peptides selected from the group consisting of peptides comprising at least 9 consecutive aminoacids comprised within anyone of the following set of peptides: set 1 consisting of SEQ ID NO 104 to 122, set 2 consisting of SEQ ID NO 123 to 142, set 3 consisting of SEQ ID NO 143 to SEQ ID NO 157, set 4 consisting of SEQ ID NO 158 to SEQ ID NO 172.

57. The composition according to claim 56 wherein said tumors are selected from the group consisting of: melanoma, hepatocarcinomas, prostatic tumors, hesophageal tumors or tumors overexpressing at least one of the markers selected from: MAGE, PSA, NY-ESO-1, or AFP.

58. A kit to perform the immunodiagnostic method according to claims 1-16 comprising at least one of the composition according to claims 26-57 and further comprising, optionally in a freeze-dried form, at least one of the following components:
    - preparation of negative and positive control related to the specific antigenic composition
    - reagents, such as solution of washing and permeabilization,
    - reagents, such as mixtures of monoclonal antibodies,
    - pipettes and other laboratory material,
    - an instruction leaflet to perform the method according to claims 1-16.

59. The kit according to claim 58 for the combined immunodiagnosis of respiratory infection comprising at least one of the compositions according to claims 31-39 and further comprising at least one of the purified proteins or antigen lysates selected in the group consisting of: Influenza A virus (H3N2) antigen lysate, Influenza A virus (H1N1) antigen, Influenza B virus (Hong Kong) antigen lysate, Influenza B virus (Victoria) antigen lysate, Influenza B virus (Tokio) antigen lysate, Influenza B virus (Qiengdao) antigen lysate, Influenza B virus (Lee) antigen lysate, Parainfluenza virus (group 1) antigen lysate, Parainfluenza virus (group 2) antigen lysate, Parainfluenza virus (group 3) antigen lysate, Parainfluenza virus (group 4) antigen lysate, Respiratory Syncytial Virus (RSV, ceppo A2) antigen lysate, SARS coronavirus recombinant protein E, SARS coronavirus recombinant protein M, SARS coronavirus recombinant protein Nucleocapsid aa.1-49, SARS coronavirus recombinant protein Nucleocapsid aa.192-220, echovirus 11 antigen lysate, Coxsackie B6 antigen lysate, Coxsackie A9 antigen lysate, Coxsackie A16 antigen lysate, adenovirus (tipo 3) antigen lysate, adenovirus (tipo 6) antigen lysate, adenovirus (tipo 21) antigen lysate, *Legionella pneumophila* antigen (Trinity Biotech Plc, Wicklow, Ireland), *Mycoplasma pneumoniae* antigen lysate, *Chlamidia pneumoniae*, antigen lysate.

60. The kit according to claim 58 for the combined immunodiagnosis of enteric infections comprising the composition according to claim 47.

61. The kit according to claim 58 for the combined immunodiagnosis of sexually transmitted diseases comprising at least one of the compositions according to claims 16-17 and further comprising at least one of the purified proteins or antigen lysates selected in the group consisting of: *Treponema pallidum* p15 recombinant antigen, *Treponema pallidum* p17 recombinant antigen, *Treponema pallidum* p45 recombinant antigen, *Treponema pallidum* TmpA recombinant antigen, HPV L1 capsid antigen recombinant protein, *Candida albicans* mixed antigen, HSV2 antigen lysate, HBV HBeAg recombinant antigen, HBV Core recombinant antigen, HBV HBsAg recombinant antigen, HIV-1 antigen lysate, HIV-2 antigen lysate, HIV-1 recombinant protein Gag, HIV-1 recombinant protein Nef, HIV-1 recombinant protein Env.

62. The kit according to claim 58 for the combined immunodiagnosis of *in utero* infections comprising at least one of the compositions according to claims 26-27 and further comprising at least one of the purified proteins or antigen lysates selected in the group consisting of: Toxoplasma gondii lysate, Toxoplasma gondii Tachyzoites antigen, Rubella recombinant protein, CMV (AD169) antigen lysate, CMV (AD169) pp65 recombinant protein, CMV (AD169) pp150 recombinant, CMV (AD169) pp28 recombinant protein, CMV (AD169) pp38 recombinant protein, CMV (AD169) p50 recombinant protein, CMV (C194) gB recombinant protein, HSV-1 gD recombinant protein, HSV-1 gG recombinant protein, HSV-1 viral lysate, VZV antigen lysate.

63. The kit according to claim 58 for the combined immunodiagnosis of post–transplant infections comprising at least one of the compositions according to claims 26-27 and further comprising at least one of the purified proteins or antigen lysates selected in the group consisting of: CMV (AD169) antigen lysate, CMV (AD169) pp65 recombinant protein, CMV (AD169) pp150 recombinant, CMV (AD169) pp28 recombinant protein, CMV (AD169) pp38 recombinant protein, CMV (AD169) p50 recombinant protein, CMV (C194) gB recombinant protein, HSV-1 gD recombinant protein, HSV-1 gG recombinant protein, HSV-1 viral lysate, EBV (B95-8) antigen lysate.

64. The kit according to claim 58 for the combined immunodiagnosis of blood-borne infections comprising at least one of the compositions according to

claims 27-28 in combination with at least one of the purified proteins or antigen lysates selected in the group consisting of: HIV-1 antigen lysate, HIV-2 antigen lysate, HIV-1 recombinant protein Gag, HIV-1 recombinant protein Nef, HIV-1 recombinant protein Env, HCV Core recombinant protein, HCV p22 nucleocapsid recombinant protein, HCV NS3 recombinant protein, HCV NS4 recombinant protein, HBV HBeAg recombinant antigen, HBV Core recombinant antigen, HBV HBsAg recombinant antigen, HDV delta antigen, recombinant, HGV antigen, recombinant, HHV-8 antigen lysate.

65. The kit according to claim 58 for the combined immunodiagnosis of threat-agent infections comprising at least one of the compositions according to claims 29-43 in combination with at least one of the purified proteins or antigen lysates selected in the group consisting of: Plague (*Yersinia pestis*) Capsular F1 antigen, Tularemia (*Francisella tularensis*) LPS antigen.

66. The kit according to claim 58 for the combined immunodetection of neoplasy comprising at least one of the compositions according to claims 56-57.

67. Use of anyone of the composition according to claims 26-57 for immunodiagnosis of T-lymphocyte activation by flow cytometry.

68. Use of the composition according to claims 27-41 for the *in vitro* diagnosis of infectiuos diseases.

69. Use of the composition according to claims 31-43 for the *in vitro* diagnosis of biological threat agents infection.

70. Use of the composition according to claims 56-57 for the *in vitro* diagnosis of tumors.

71. The use according to claims 56-57 for the follow up of a chemotherapeutic treatment.

72. Use of the composition according to claim 62 for the *in vitro* diagnosis of in utero infections

73. Use of the composition according to claim 63 for the *in vitro* diagnosis of post transplant infections.